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## Virus Vaccine

The present invention relates to a virus vaccine, specifically a vaccine to Venezuelan equine encephalomyelitis virus (VEE), to its preparation and pharmaceutically acceptable formulations and methods of prophylactic and therapeutic methods of treatment using said vaccine.

VEE virus is a mosquito-borne alphavirus which is an important cause of epidemic disease in humans and of epizootics in horses, donkeys and mules in certain parts of the world, in particular the South Americas.

The existing VEE vaccine, TC-83, was initially produced by attenuation of the Trinidad donkey strain (TRD) of VEE by sequential passage in guinea pig heart cell cultures. However, this vaccine is generally regarded as being inadequate for human vaccination. This is mainly due to the high incidence of side effects in vaccinees and the large proportion of vaccinees who fail to develop neutralising antibodies (Monath et al. 1992, Vaccine Research, 1, 55-68).

A vaccinia-based vaccine against VEE has been constructed (Kinney et al. J. Gen. Virol. 1988, 69, 3005-3013). In this recombinant, 26S RNA encoding structural genes of VEE were inserted into the NYCBH strain of vaccinia. The recombinant virus protected against sub-cutaneous challenge but had limited efficacy against aerosol challenge with VEE.

The virulent Trinidad donkey strain of VEE and the attenuated strain TC-83 have both been cloned and sequenced (R.M. Kinney et al. Virology (1989) 170, 19-30) and the amino acid and nucleotide numbering system used in this reference will be used hereinafter. This work has revealed that there are a number of amino acid changes between TRD and TC-83. The majority (five) of these changes occur within the gene encoding the glycoprotein E2.

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The changes have been summarised as follows:

		Table 1			
		Nucleotide		Amino acid	
5	change				
	Position	TRD	TC-83	TRD	TC-83
	22, junction region	A	G	non-coding	
	1053, E <sub>2</sub> -7	G	U	Lys	Asn
	1285, E <sub>2</sub> -85	C	U	His	Tyr
	1391, E <sub>2</sub> -120	C	U	Thr	Arg
	1607, E <sub>2</sub> -192	U	A	Val	Asp
	1866, E <sub>2</sub> -278	U	C	none	
	1919, E <sub>2</sub> -296	C	U	Thr	Ile
	2947, E <sub>1</sub> -161	U	A	Leu	Ile
	3099, E <sub>1</sub> -211	A	U	none	
	3874, 3'-non-coding region	UU	U	non-coding	

It has also been shown that the first 25 amino acids of the E2 glycoprotein represents a protective epitope. This region includes a single amino acid change (lys → asp) at amino acid 7 in the TC-83 construct as compared to the TRD strain. A 25bp synthetic peptide based on the TRD sequence VE2pep01 (TRD), protected more mice from TRD virus challenge than a corresponding TC-83 based peptide (A.R. Hunt et al., Virology, 1990, 179, 701-711). More precise mapping of this epitope has been carried out (A.R Hunt et al., Vaccine 1995, 13, 3, 281-288).

The applicants have found ways of increasing the protectiveness of a vaccine and in particular a vaccinia-based vaccine.

In particular, the applicants have found that the protectiveness of the vaccine may be increased either (a) by restoring the lysine residue at amino acid 7 of the E2 protein and/or (b) by modifying the promoter to increase expression of the protective construct.

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Thus, in a first aspect, the present invention provides a vaccine for the therapeutic or prophylactic immunisation against Venezuelan Equine Encephalitis (VEE) virus, said vaccine

comprising a vector which includes a sequence which encodes an attenuated form of said virus which is capable of producing a protective immune response, wherein the said sequence is such that the amino acid at position 7 in the E2 protein of VEE is lysine.

Suitably, the attenuated form of the VEE virus comprises a derivative or variant of the TC-83 construct or an immunogenic fragment thereof.

10 Other attenuated forms may be produced by the skilled person, for example using known techniques such as serial passage through another organism, or by recombinant DNA technology, for instance by inactivating genes associated with the replication or virulence of the virus. The structural gene encoding the E2

15 glycoprotein or a fragment encoding at least the N-terminal 19 amino acids should be retained in order to retain immunogenicity of the construct.

Suitable fragments of the construct are those which include only some of the structural genes of the VEE peptide or which encode only part of the proteins encoded by said genes, provided the construct encodes sufficient antigenic determinants to ensure that it is capable of producing a protective immune response in a mammal to whom the construct is administered.

25 As used herein, the term "variant" means that the construct is different to the original strain but that it encodes proteins and/or peptides which are the same or similar to those of wild-type VEE or immunogenic fragments thereof.

30 Thus, the changes in the nucleotide sequence may be silent in that they do not produce amino acid changes as compared to the original strain, or they may produce amino acid changes provided these do not alter function of the construct in terms of its ability to produce a protective immune response against VEE. For example, the construct may encode peptides

35 or proteins which are 60% homologous to the wild-type proteins or peptides, suitably more than 80% homologous and preferably more than 90% homologous to the native protein sequence, and provided they produce antibodies which are cross-reactive with

40 wild-type VEE, the protective effects of the construct may be retained.

"Derivatives" may have broadly similar structures but they are derived by manipulating the original constructs using recombinant DNA technology or chemical modification if appropriate.

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The vector may contain the usual expression control functions such as promoters, enhancers and signal sequences, as well as a selection marker in order to allow detection of successful transformants. The selection of these will depend upon the precise nature of the vector chosen and will be known to or readily determinable by a person skilled in the art.

Suitably the vector is a viral vector, for example a vector derived from vaccinia, adenovirus, or herpes simplex virus (HSV) BCG or BCC. It is suitably attenuated itself, to minimise any harmful effects associated with the virus on the host.

Preferably, the vector is derived from vaccinia virus, as it has many properties which make it a suitable vector for vaccination, including its ability to efficiently stimulate humoral as well as cell-mediated immune responses. Vaccinia has proven utility as a vaccine vehicle, following the Smallpox eradication programmes. It provides the potential for multi-valent vaccine construction and for oral administration. There are many attenuated strains currently available.

A suitable selection marker for inclusion in a vaccinia vector is the gpt marker gene.

A VEE vaccine was constructed using a WR strain of vaccinia in this work. Preferably, a more highly attenuated strain of vaccinia which would be more acceptable for use in humans is employed. Such strains include Lister, which was used for wide scale vaccination against smallpox, NYVAC (Tartaglia et al, (1992). *AIDS Research and Human Retroviruses* 8, 1445-1447) which contains specific genome deletions, or MVA (Mayr et al, (1975) *Infection* 3, 6-14) which is also highly attenuated.

Vaccines based upon viral vectors are suitably formulated for parenteral administration as described above. However, it is possible to formulate such vaccines for oral administration, for

example by incorporating the vector into a gut-colonising microorganism such as *Salmonella* and particularly *S. typhimurium*.

- 5 pTC-5A is a plasmid clone of cDNA encoding the structural genes of VEE virus strain TC-83 (Kinney et al. J. Gen. Virol. (1988) 69, 3005-30130). The VEE cDNA is situated downstream of the vaccinia 7.5K promoter which drives expression of the VEE structural proteins when the plasmid is used to construct recombinant vaccinia viruses.
- 10 Modified 7.5K vaccinia promoters have previously been prepared (Davison & Moss, J. Mol. Biol. 210, (1989) 749-769). It has been found that certain substitution mutations increase the strength of the promoter. By using synthetic promoters which include substitution mutations, the amount of VEE proteins produced from the recombinant
- 15 virus was increased.

Thus in a further aspect of the invention, there is provided a vaccine for the therapeutic or prophylactic immunisation against Venezuelan Equine Encephalitis (VEE) virus, said vaccine

20 comprising a vaccinia virus vector which encodes an attenuated form of the VEE virus or a variant or fragment thereof which is capable of producing a protective immune response against VEE virus, expression of the said attenuated VEE virus being under the control of a synthetic 7.5K vaccinia promoter which has been

25 subject to mutation which increases the level of VEE virus protein production as compared to the wild-type 7.5K promoter.

In particular, it has been found that substitution mutations within the 7.5Kd promoter can be effective. These may be

30 illustrated by the following Table:

Wild-type 7.5K promoter:

TAAAAGTAGAAAATATATTCTAATTTATTGCAC (SEQ ID No 1)

35

Substitution Mutations (emboldened)

TAAA**A**ATTGAAAATACATTCTAATTTATTGCAC (SEQ ID No 2)

TAAA**A**ATTGAAAATATATTCTAATTTATTGCAC (SEQ ID No 3)

40

Inclusion of a synthetic 7.5K vaccinia promoter in WR103 has

been found to increase expression of the downstream VEE cDNA, leading to a 3.59-fold increase in protein production.

The vaccine may comprise the vector itself but it is suitably formulated as a pharmaceutical composition in combination with a pharmaceutically acceptable carrier or excipient. Such compositions form a further aspect of the invention. The compositions may be in a form suitable for oral or parenteral application.

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Suitable carriers are well known in the art and include solid and liquid diluents, for example, water, saline or aqueous ethanol. The liquid carrier is suitably sterile and pyrogen free.

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The compositions may be in the form of liquids suitable for infusion or injection, or syrups, suspensions or solutions, as well as solid forms such as capsules, tablets, or reconstitutible powders.

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Constructs for use in the vaccines of the invention may be prepared by various means as will be understood in the art, ranging from modification of available constructs such as the wild-type virus using recombinant DNA technology or by synthetic means. Recombinant DNA techniques include site directed mutagenesis, optionally involving PCR amplification as illustrated hereinafter.

As illustrated hereinafter, recombinant vaccinia virus was constructed which expressed the structural genes of VEE as produced by a modified form of TC-83. The ability of the recombinant virus to elicit protective immune responses against virulent VEE disease was investigated.

35 In yet another embodiment, the vaccine further comprises a cytokine or an active fragment or variant thereof. The cytokine may itself be incorporated into the vaccine formulation, or more suitably, the vector may include a coding sequence which means that the cytokine is co-expressed by the vector. Examples of  
40 suitable cytokines include interleukin 2 (IL-2) and interleukin 6 (IL-6).

A particularly suitable cytokine is interleukin 2 (IL-2), which may be expressed from for example a vaccinia virus recombinant. IL-2 is known to be responsible for the clonal expansion of antigen-activated T cells (Smith, (1984) *Reviews in Immunology* 2, 319-333).

Alternatively, antibody levels can be enhanced using other cytokines. For example, expression of IL-6 by vaccinia vectors has been shown to induce a high level of IgG<sub>1</sub> (Ruby et al, 1992 *Vaccine Research* 1, (4), 347-356), and IL-5 and IL-6 induced mucosal IgA responses to co-expressed influenza HA (Ramsay et al, (1994) *Reproduction, Fertility and Development* 6, 389-392).

The vaccine of the present invention may be used to treat humans or animals. In particular it may be given to horses, as a veterinary vaccine, to prevent infection, or as a prophylactic or therapeutic vaccine for humans.

The vaccine of the invention may be incorporated into a multivalent vaccine in order to increase the benefit-to-risk ratio of vaccination.

The dosage of the vaccines of the invention will depend upon the nature of the mammal being immunised as well as the precise nature and form of the vaccine. This will be determined by the clinician responsible. However in general, when using a virus vector such as a vaccinia virus vectors, dosages of the vector may be in the range of from  $10^4$ - $10^{12}$ pfu (pfu = particle forming units).

The vaccines of the invention will produce an immune response in test animals including the production of antibodies. These antibodies may be useful in passive vaccination programmes or in diagnosis of VEE virus disease. For diagnostic purposes, the antibodies may form part of a kit as is conventional in the art.

The invention will now be illustrated by way of Example with reference to the accompanying drawings in which

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Figure 1 shows the construction of chimeric plasmids used for

generation of recombinant vaccinia viruses;

Figure 2 shows the results of a immunofluorescence assay using polyclonal antiserum to TC-80;

5

Figure 3 is a graph showing the results of an experiment to quantify by ELISA the amount of VEE protein expressed by strains; and

10 Figure 4 is a graph showing the results of an experiment to find the level of anti-VEE IgG in animals vaccinated with various strains of the invention.

15 In the Examples, relative protein levels were calculated from ELISA data using regression analysis performed by Minitab statistical analysis software (Minitab Inc., State College, PA, USA). Serum antibody levels were compared by the two sample t test. Contingency tables were analysed by Fisher's exact test. P values of <0.05 were taken to be significant.

20

#### Example 1

##### Alteration of the E2 protein sequence

pTC-5A, a plasmid clone of cDNA encoding the structural genes of Venezuelan Equine Encephalitis virus, strain TC-83 was  
25 obtained from Dr. R. Kinney (Kinney et al, 1988, Journal of General Virology 69, 3005-3103). An Eco RI fragment containing the VEE cDNA was removed from pTC-5A and inserted into p1113 (Carroll, 1993, Ph.D. thesis, Faculty of Medicine, University of Manchester; Fig 1a) which is a shuttle vector  
30 used for insertion of genes into the thymidine kinase locus of vaccinia with dominant selection of recombinant viruses based on resistance to mycophenolic acid (Falkner & Moss, 1988, Journal of Virology 62, 1849-1854). The resulting plasmid, pAB100, was mixed with Lipofectin™ (Life Technologies) and  
35 used to transfect CV-1 cells infected with vaccinia virus, strain WR. Recombinant viruses were designated WR100 and were subjected to three rounds of plaque-purification before preparation of stocks as described earlier (Mackett et al, 1985 DNA cloning (Volume II): a practical approach).

40

The sequence of VEE E2, strain TC-83, situated in pTC-5A, was



altered by one nucleotide substitution from T to G as position 1053 as compared to wild-type VEE TRD (Johnson et al. J. Gen. Virol. 1986, 67, 1951-1960). This resulted in an amino acid change from asparagine to lysine in the E2 protein when expressed from the vaccinia virus.

In order to perform this particular amino acid change, the following manipulations were carried out.

A cleavage site for restriction enzyme *Nsi I* occurs close to the site of the required nucleotide substitution. A second *Nsi I* site is situated about 500bp upstream. Oligonucleotide primers were used to amplify the DNA sequence between the *Nsi I* sites using the Polymerase Chain Reaction (PCR). The downstream primer contained a nucleotide mismatch corresponding to the TRD sequence at this point.

The primer sequences are listed below. The *Nsi I* cleavage sites and the position of the substituted nucleotide are underlined.

Primer 1 designated "Nsi 1"

5' GCC GAT GCA TGT GGA AGG C 3'

Primer 2 designated "Nsi 2"

5' ATC TGA TGC ATC TGG CCA TGT AAG GGC GCG TTA GCT TAT  
ACT CQT TAA ACA GC 3'

The PCR product was digested with *Nsi I* and used to replace the corresponding *Nsi I* fragment in pTC-5A, generating plasmid pAB101. The nucleotide sequence of the relevant region in pAB101 was obtained to verify the sequence alteration.

pAB101 was then digested with *Eco RI* to remove the VEE 26S RNA coding sequence which was transferred to the vaccinia shuttle vector plasmid p1113. P1113 contains the selectable marker *gpt* which allows selection of recombinant vaccinia viruses. The plasmid constructed by the addition of the VEE sequence to p1113 was designated pAB102.

**Example 2****Substitution of 7.5K promoter for a synthetic promoter in pAB102**

A synthetic 7.5K vaccinia promoter was designed, based upon work by Davison and Moss (supra.). Complementary oligonucleotides  
5 were designed with 5' *Bam* HI and 3' *Eco* RI ends. The oligonucleotides were annealed and ligated into the plasmid pT7Blue (available from AMS Biotechnology (UK) Ltd). The plasmid clone was digested with *Bam* HI and *Eco* RI and the DNA fragment containing the synthetic promoter was isolated and cloned into  
10 the plasmid pAB102 which had been cut with the same enzymes. This resulted in the generation of plasmid pAB103 (Figure 1) which contains the synthetic promoter upstream of the VEE 26S RNA coding sequence. Vaccinia WR strain was transformed with pAB103 to produce the recombinant vaccinia virus WR103.

15

The sequence of the oligonucleotides used is given below. Substitutions in the 7.5K promoter sequence are given in bold type. Insertions are underlined. Oligonucleotide "tails" containing restriction enzyme cleavage sites are italicised.

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**Oligo 1 designated "7.5KF2"**

5'     ACG CGG ATC CAA AAA TTG AAA AAC TAG CTT AAA AAT TGA  
AAA ACT ATT CTA ATT TAT TGC ACG AAT TCC G     3'

25

**Oligo 2 designated "7.5KR2"**

This is the reverse complement of 7.5KF2.

30

The amount of VEE proteins produced by the recombinant virus WR103 was measured using enzyme linked immunoabsorbant assay (ELISA).

**Example 3****Analysis of protein expression**

35

VEE viral proteins were visualised by indirect immunofluorescence of infected CV-1 cells. CV-1 monolayers (25cm<sup>2</sup>) were infected with virus at a multiplicity of 2 p.f.u. per cell. At 24 hours post infection, cells were scraped into the growth media and washed once with phosphate-buffered saline  
40 (PBS) containing 0.1% bovine serum albumin. Cells were spotted onto slides, air-dried and fixed in acetone. Binding

of mouse polyclonal antiserum raised against VEE strain TC-80 (provided by Dr. A.D.T. Barrett, University of Texas) was detected with fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Amersham International plc).

5

Examination of cells infected with WR100 or WR103 showed that WR103-infected cells fluoresced more brightly than the WR100-infected cells (Figure 2).

10

Quantification of VEE viral protein expression was carried out using an enzyme-linked immunosorbent assay (ELISA). CV-1 monolayers (150 cm<sup>2</sup>) were infected with virus at a multiplicity of 10 p.f.u. per cell and harvested at 24 hours post infection by scraping into the growth media. Cells were washed once in

15

PBS and resuspended in T9 buffer (10 mM Tris.HCl; 1 mM EDTA; pH 9.0). Samples were frozen, thawed and sonicated for 1 minute in a sonicating bath. Cells debris was pelleted for 5 minutes at 1800 g and the supernatant was centrifuged for 30 minutes at 10,000 x g. The supernatant was removed and stored

20

at -70°C. The cell lysate preparation was diluted 1/30 in bicarbonate buffer (Sigma), 100  $\mu$ l volumes were added to wells of a microtitre plate and the antigen was allowed to bind at 37°C for 1 hour. Lysates were replaced with 200  $\mu$ l/well of saline containing 10% formaldehyde. Plates were incubated at

25

room temperature for 20 minutes, then washed 6 times with PBS containing 0.1% Tween (PBST). Mouse polyclonal anti-TC80 was serially diluted in blocking solution (0.5% dried milk/PBST), added to wells, and the plates were incubated for 1 hour at 37°C. Plates were washed 3 times in PBST before addition of

30

horseradish peroxidase-conjugated mouse specific antibody (diluted 1:1000 in blocking solution) and incubated for 1 hour at 37°C. Plates were washed 3 times before addition of ABTS in citrate buffer and incubation at room temperature for 1 hour. Colour development was measured at A<sub>414</sub>.

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This quantification process revealed that WR103-infected cells contained 3.59-fold more VEE protein than WR100-infected cells (Figure 3).

40

Quantification of vaccinia protein in these samples had demonstrated equivalent amounts in each (data not shown), so

12.

it must be assumed that the difference in VEE protein content is due to different expression levels of the encoded VEE cDNA.

#### 5 Example 4

##### Protective effect of Vaccinia recombinants

Groups (10) of female 6-8 week old Balb/c mice were inoculated with PBS or with  $10^8$  p.f.u. of vaccinia viruses by intramuscular injection, or with  $10^5$  p.f.u. of TC-83 by subcutaneous injection. Serum was taken for measurement of immunoglobulins to VEE proteins.

The vaccinated mice were challenged with two different doses of virulent VEE strain TRD at 35 days after immunisation. The survival rates after 14 days are presented in Table 2.

Table 2

Strain	10pFu TRD	100 pFu TRD
WR	0/10	0/10
WR100	1/10	2/10
WR103	6/10	6/10
No treatment	0/10	0/10

20 WR100: Vaccinia/VEE recombinant

WR103: Vaccinia/VEE recombinant produced in Example 2 above.

These results show that genetic manipulation of the recombinant virus has improved the protection afforded by the construct. A significant improvement in protection of mice following subcutaneous challenge with TrD was seen when WR103 was used for vaccination, compared with WR100 ( $P < 0.05$ , Table 2). WR100 protected up to 20% of mice whereas WR103 protected 60% of mice. There was not a significant difference between numbers of mice protected when challenge doses of 10 p.f.u. or 100 p.f.u. of TrD were used. The challenge dose had previously been titrated to show that 1 p.f.u. of TrD approximates to 2-3 LD<sub>50</sub> doses (data not shown).

**Example 5****Immunoassays**

VEE virus-specific immunoglobulin in serum was measured by enzyme-linked immunoassay as follows. Wells of a microtitre plate were coated with purified TC-83 at 37°C for 1 hour. Serum was diluted serially in blocking solution and allowed to bind to antigen-coated wells overnight at 4°C. Plates were washed 3 times and incubated with horseradish peroxidase-conjugated anti-mouse immunoglobulin at 37°C for 1 hour. Plates were washed and incubated with TMB substrate for 20 minutes before measurement of colour development at  $A_{450}$ .

All vaccinia-inoculated mice responded to the vaccination by the detection of immunoglobulin to vaccinia virus in serum (data not shown). Immunoassay to measure TC-83 antibody failed to detect anti-VEE IgG in WR100 samples. WR103 samples contained a detectable level of anti-VEE antibody although this was substantially lower than the amount found in serum from mice vaccinated with TC-83 (Figure 4).

Neutralising antibody was measured by a plaque reduction test. Serum (10  $\mu$ l) was incubated with TC-83 (50  $\mu$ l) and maintenance medium (140  $\mu$ l) for 1 hour at room temperature. Maintenance medium (800  $\mu$ l) was added and the suspension was used to infect confluent monolayers of BHK-21 cells grown in 6-well plates. Plates were incubated at 37°C for 3 days. A 50% reduction in the number of plaques per well, compared to control wells, was indicative of the presence of neutralising antibody.

Neutralising antibody to TC-83 was found in serum from mice vaccinated with TC-83 but was not detected in serum from mice vaccinated with WR100 or WR103 (data not shown). Although neutralising antibody is usually found in mice which are protected against VEE challenge, protection has previously been reported in the absence of detectable neutralising antibody (Kinney et al, 1988a, Journal of Virology 62, 4697-4702).

## Claims

1. A vaccine for the therapeutic or prophylactic immunisation against Venezuelan Equine Encephalitis (VEE) virus, said vaccine comprising a vector which includes a sequence which encodes an attenuated form of said virus which is capable of producing a protective immune response, wherein the said sequence is such that the amino acid at position 7 in the E2 protein of VEE is lysine.
2. A vaccine according to claim 1 wherein the attenuated form of said virus comprises a derivative of the TC-83 construct.
3. A vaccine according to claim 2 wherein the vector comprises a virus vector.
4. A vaccine according to claim 3 wherein the virus is selected from an attenuated virus
5. A vaccine according to claim 3 or claim 4 wherein the virus is selected from vaccinia, adenovirus, HSV, BCG or BCC.
6. A vaccine according to claim 5 which comprises an attenuated vaccinia virus.
7. A vaccine according to claim 6 wherein expression of the said attenuated VEE virus is under the control of a synthetic 7.5K vaccinia promoter which has been subject to mutation which increases the level of VEE virus protein production as compared to the wild-type 7.5K promoter.
8. A vaccine according to claim 7 wherein the said 7.5K promoter comprises a sequence selected from
- TAAAAATTGAAAATACATTCTAATTTATTGCAC (SEQ ID No 2)
- or
- TAAAAATTGAAAATATATTCTAATTTATTGCAC (SEQ ID No 3).
9. A vaccine according to any one of the preceding claims which comprises a vector which includes a nucleotide sequence which encodes a further immunogenic peptide, and is able to express said sequence when administered to a mammal.

10. A vaccine according to any one of the preceding claims which further comprises a cytokine or an active fragment or variant thereof, or a vector which comprises a nucleotide sequence which encodes a cytokine or an active fragment or variant thereof.

11. A vaccine according to claim 10 which comprises a vector which comprises a nucleotide sequence which encodes a cytokine or an active fragment or variant thereof.

12. A vaccine according to claim 10 or claim 11 wherein the cytokine is an interleukin.

13. A vaccine according to claim 10 wherein the interleukin is selected from human IL-2 or human IL-6.

14. A vaccine for the therapeutic or prophylactic immunisation against Venezuelan Equine Encephalitis (VEE) virus, said vaccine comprising a vaccinia virus vector which encodes an attenuated form of the VEE virus or a variant or fragment thereof which is capable of producing a protective immune response against VEE virus, expression of the said attenuated VEE virus being under the control of a synthetic 7.5K vaccinia promoter which has been subject to mutation which increases the level of VEE virus protein production as compared to the wild-type 7.5K promoter

15. A pharmaceutical composition comprising a vaccine as defined in any one of the preceding claims and a pharmaceutically acceptable carrier or excipient.

16. A method for producing a protective immune response against VEE virus in a mammal, which method comprises administering to said mammal, a vaccine according to any one of claims 1 to 14.

17. A method according to claim 16 wherein the mammal is either a human or a horse.

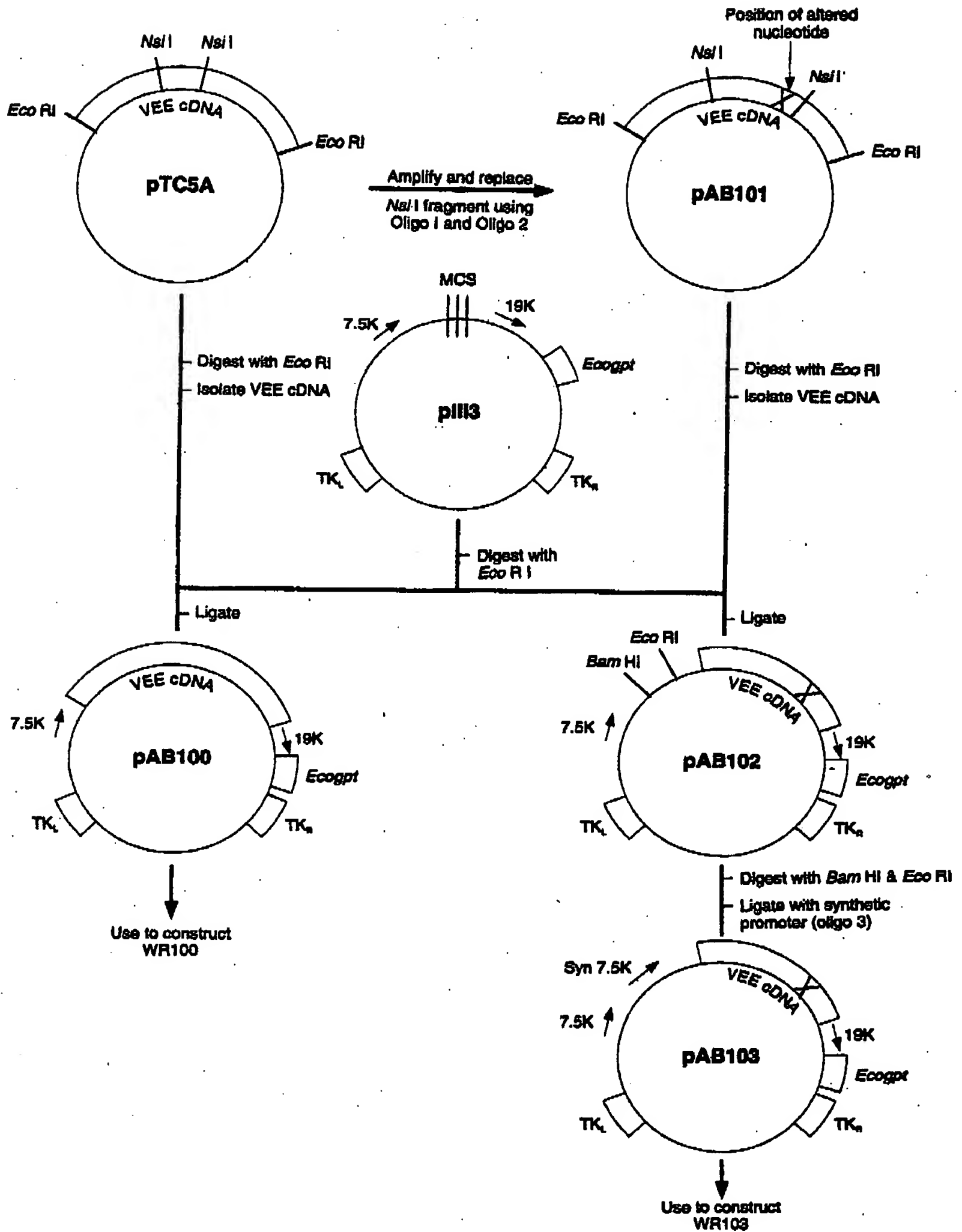
18. A multivalent vaccine comprising a vaccine according to any one of claims 1 to 14 and a further vaccine.

**Abstract**

A prophylactic or therapeutic vaccine for use in protecting mammals such as humans or animals against Venezuelan Equine Encephalitis virus (VEE) is described. In particular, the vaccine comprises a recombinant virus such as a recombinant vaccinia virus which is able to express the structural genes of VEE in attenuated form, which has been modified to increase the protective effect of the vaccine. This is achieved by modifying the sequence of the attenuated VEE strain and/or putting this under the control of modified promoter which increases expression from the vector.

Formulations of the vaccine as well as methods of treatment using the vaccine are also described.





**Fig. 1(a) Construction of chimeric plasmids used for generation of recombinant vaccinia viruses**

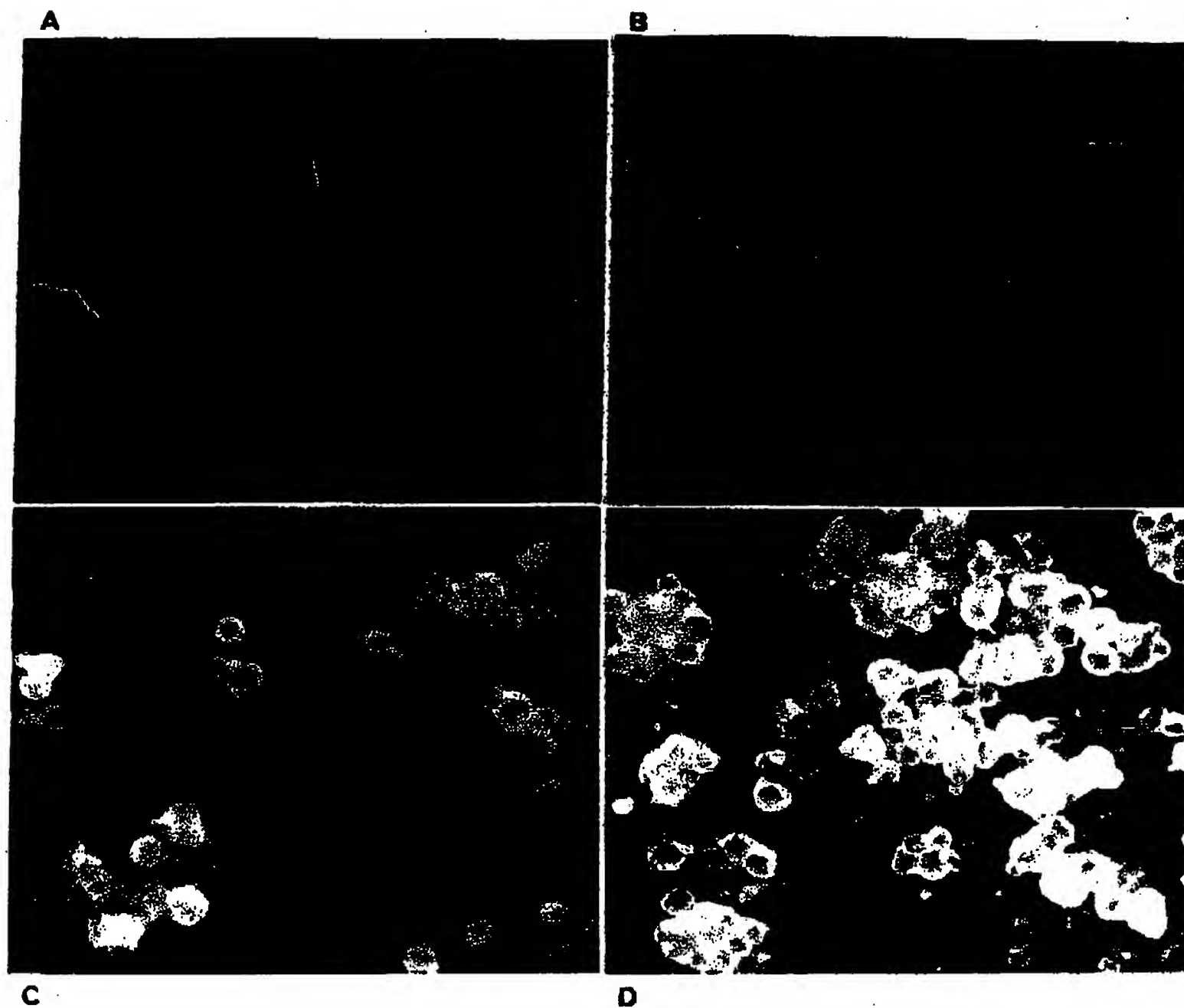


FIGURE 2

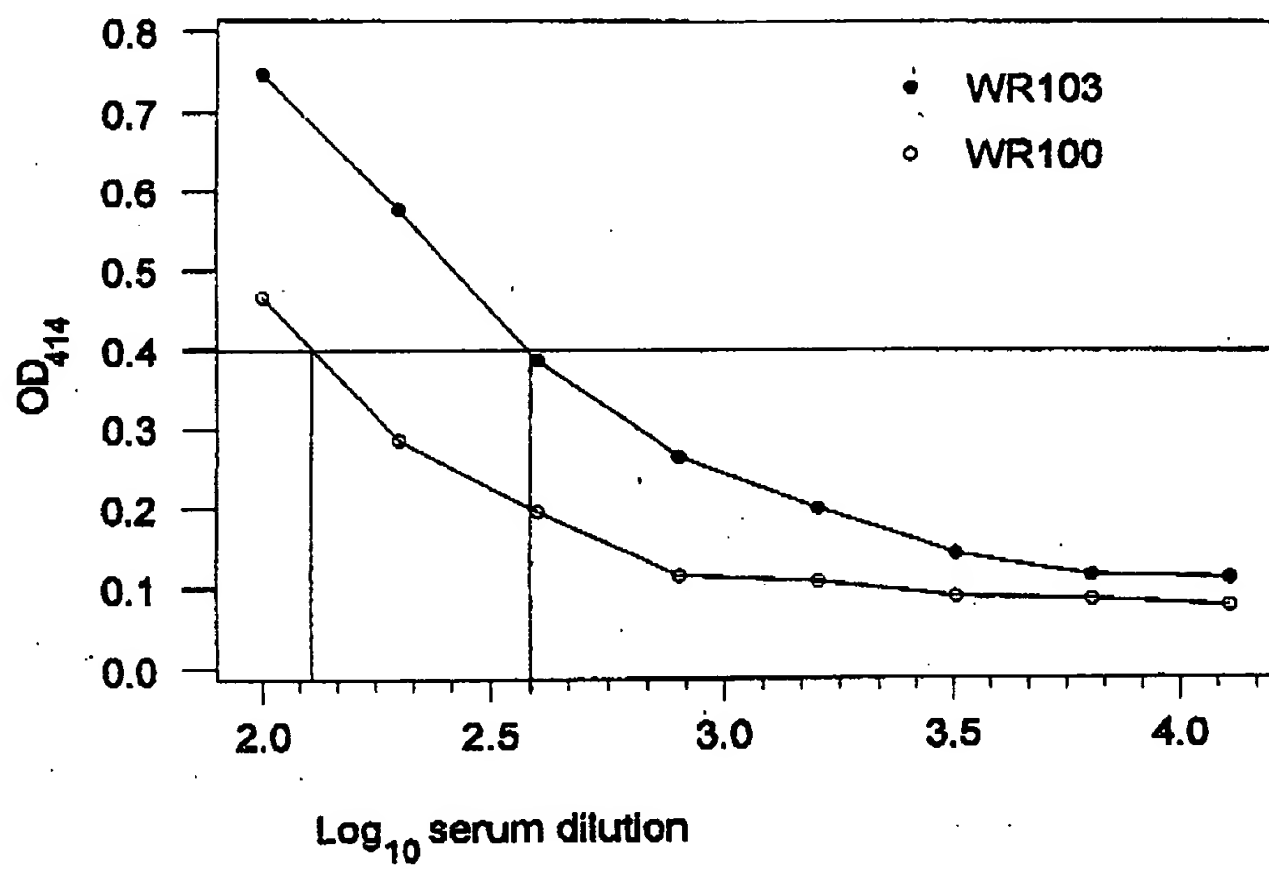


FIGURE 3

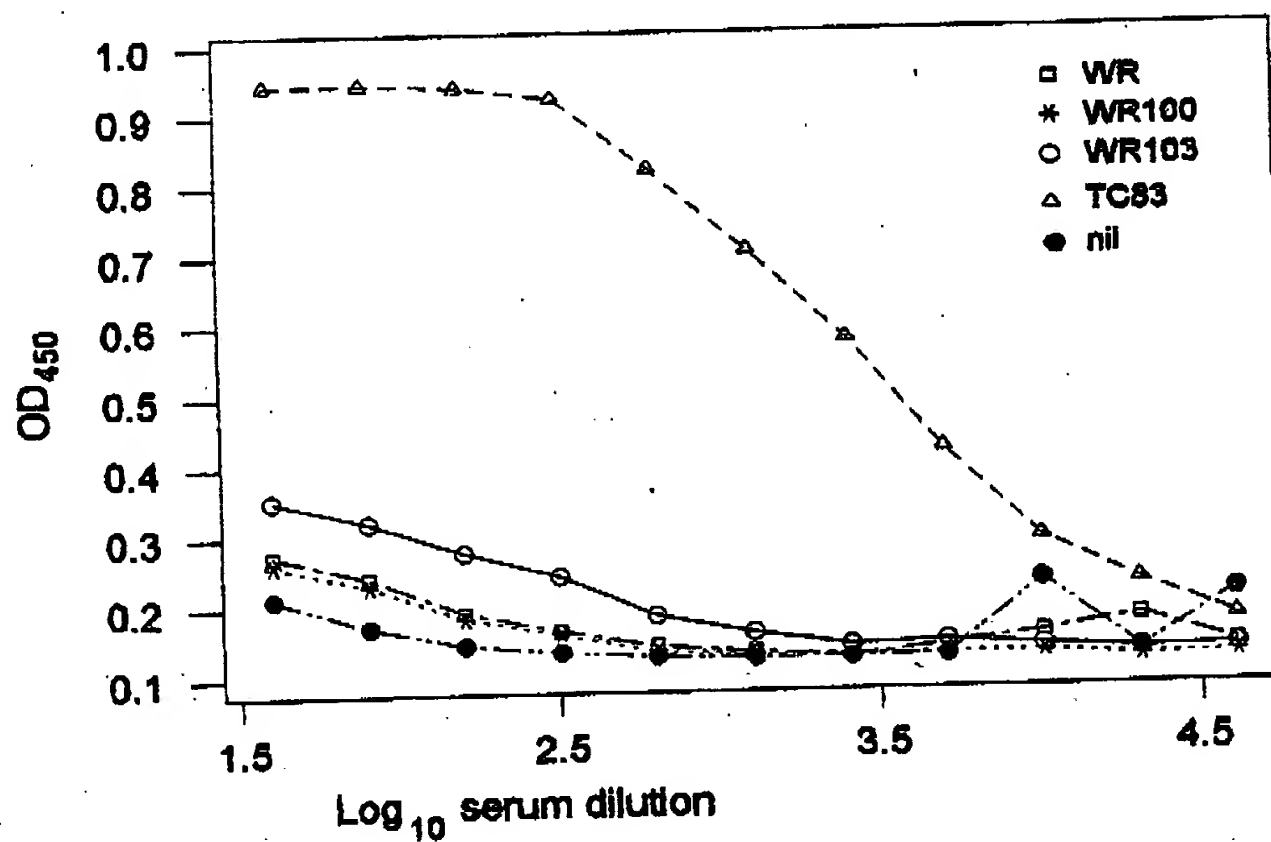


FIGURE 4

XP ∞ 2117670

A

# The Full-Length Nucleotide Sequences of the Virulent Trinidad Donkey Strain of Venezuelan Equine Encephalitis Virus and Its Attenuated Vaccine Derivative, Strain TC-83

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Nucleotide sequence analysis of cDNA clones covering the entire genomes of Trinidad donkey (TRD) Venezuelan equine encephalitis (VEE) virus and its vaccine derivative, TC-83, has revealed 11 differences between the genomes of TC-83 virus and its parent. One nucleotide substitution and a single nucleotide deletion occurred in the 5'- and 3'-noncoding regions of the TC-83 genome, respectively. The deduced amino acid sequences of the nonstructural polypeptides of the two viruses differed only in a conservative Ser (TRD) to Thr (TC-83) substitution in nonstructural protein (nsP) three at amino acid position 260. The two silent mutations (one each in E1 and E2), one amino acid substitution in the E1 glycoprotein, and five substitutions in the E2 envelope glycoprotein of TC-83 virus were reported previously (B. J. B. Johnson, R. M. Kinney, C. L. Kost, and D. W. Trent, 1986, *J. Gen. Virol.* 67, 1951-1960). The genome of TRD virus was 11,444 nucleotides long with a 5'-noncoding region of 44 nucleotides. The carboxyl terminal portion of VEE nsP3 contained two peptide segments (7 and 34 amino acids long) that were repeated with high fidelity. The open reading frame of the nonstructural polyprotein was interrupted by an in-frame opal termination codon between nsP3 and nsP4, as has been reported for Sindbis, Ross River, and Middelburg viruses. The deduced amino acid sequences of the VEE TRD nsP1, nsP2, nsP3, and nsP4 polypeptides showed 60-66%, 57-58%, 35-44%, and 73-71% identity with the aligned sequences of the cognate polypeptides of Sindbis and Semliki Forest viruses, respectively. The lack of homology in the nsP3 of the viruses is due to sequence variation in the carboxyl terminal half of this polypeptide.

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## INTRODUCTION

The Alphavirus genus of the Togaviridae family of viruses contains six antigenic complexes: Middelburg, Ndumu, Semliki Forest (SF), and eastern (EEE), western (WEE), and Venezuelan (VEE) equine encephalitis viruses (Calisher *et al.*, 1980). These viruses contain a positive sense, single-stranded 42S-49S RNA genome, which is capped at the 5'-terminus and polyadenylated at the 3'-terminus, enclosed within an icosahedral nucleocapsid surrounded by a lipid bilayer containing two integral glycoproteins, E1 and E2 (Kaariainen and Soderlund, 1978). A third small glycoprotein (E3) is incorporated into the envelope of mature SF virions. Alphavirus structural proteins are translated as a polyprotein precursor in the order NH<sub>2</sub>-capsid-E3-E2-6K-E1-COOH from a subgenomic 26S mRNA identical to the 3'-terminal one-third of the 42S genomic mRNA (Strauss and Strauss, 1986). The polyprotein precursor undergoes proteolytic cleavage to produce individual structural proteins. The 5'-two-thirds of the 42S RNA genome encodes a polyprotein precursor that is pro-

cessed to produce nonstructural polypeptides nsP1, nsP2, nsP3, and nsP4 which are involved in virus replication (Strauss and Strauss, 1986). The 26S mRNA equivalents of EEE (Chang and Trent, 1987) and WEE (Hahn *et al.*, 1988) have been sequenced, and the entire nucleotide sequences of the RNA genomes of Sindbis (SIN) (Strauss *et al.*, 1984; Rice and Strauss, 1981), SF (Takkinen, 1986; Garoff *et al.*, 1980a,b), and Ross River (RR) (Faragher *et al.*, 1988; Strauss *et al.*, 1988; Dalgarno *et al.*, 1983) have been determined.

VEE, EEE, and WEE viruses are mosquito-borne veterinary pathogens that produce encephalitis in equines and humans during sporadic disease outbreaks in the western hemisphere (Monath and Trent, 1981). Numerous VEE epidemics with fatality rates of 38-83% in equines have been recorded in South America (Groot, 1972). The disease is generally milder in humans. However, human fatality rates as high as 1% have been attributed to VEE virus infection in some epidemics (Groot, 1972).

Initial VEE vaccines consisted of formaldehyde-inactivated preparations of virulent virus which were not infectious for laboratory animals, although about 4% of human vaccinees developed VEE illness (Berge *et al.*,

Sequence Data from this article have been deposited with the EMBL/GenBank Data Libraries under Accession No. J04332.

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1961). A live attenuated VEE vaccine was developed by serial passage of the virulent Trinidad donkey (TRD) virus in tissue culture (Berge *et al.*, 1961). The TRD virus, which is uniformly fatal for mice by peripheral or intracranial inoculation, became avirulent for mice by either route after 45 serial passages in guinea pig heart cells. Further passage resulted in the TC-83 vaccine strain of VEE virus (Berge *et al.*, 1961; McKinney, 1972; Johnson and Martin, 1974). TC-83 virus elicits VEE-specific neutralizing antibodies in most humans (Johnson and Martin, 1974; Burke *et al.*, 1977; Fillis and Calisher, 1979) and equines (Johnson and Martin, 1974; Ferguson *et al.*, 1978) and was administered to more than two million horses in the southeastern United States after epidemic VEE virus was introduced into Texas in 1971 (Sharman, 1972; Lord, 1974).

Equine-virulent VEE strains infect the hematopoietic and lymphatic systems of the infected vertebrate host and, depending on the host species, variably affect the central nervous system (Johnson and Martin, 1974). The virus replicates in many tissues of the mouse and causes a fatal, paralytic encephalitis in animals infected by peripheral or intracranial routes (Gleiser *et al.*, 1962; Johnson and Martin, 1974). The occurrence of high viremia correlates well with encephalitis and death in horses experimentally infected with epidemic VEE virus (Walton *et al.*, 1973). Mice infected peripherally with TC-83 virus develop lower virus titers in the blood and brain (LeBlanc *et al.*, 1978). Histopathological lesions that occur in the spleen, lymph nodes, and brain of mice infected with virulent VEE virus are usually absent in TC-83 virus-infected mice (LeBlanc *et al.*, 1978). Hamsters infected subcutaneously with TC-83 virus develop a high viremia, with virus replication in the spleen, lymph nodes, bone marrow, and brain (Jahrling and Scherer, 1973a; Austin and Scherer, 1971). However, hamsters do not develop hematopoietic and neuronal histopathology typical of virulent virus infection. Avirulent VEE virus is cleared faster than virulent virus from the blood of hamsters and monkeys and is readily phagocytized by endothelial and Kupffer cells in the liver of hamsters (Jahrling and Scherer, 1973b; Jahrling and Gorelkin, 1975; Jahrling *et al.*, 1977). Clearance rate and virulence of VEE virus may be influenced by glycoprotein domains involved in virus attachment (Marker and Jahrling, 1979; Jahrling and Eddy, 1977).

The virulent VEE virus phenotype appears to be related to the level of virus replication and resulting histopathology in various tissues. Destruction of cells in the hematopoietic and lymphatic systems may compromise the immune responses of animals infected with virulent VEE virus (LeBlanc *et al.*, 1978). Virulent strains of another alphavirus, SF virus, replicate in the spleen,

liver, lymph nodes, and brains of infected mice, whereas avirulent SF strains lose the ability to damage neurons or to enter the brain (Atkins *et al.*, 1985). Avirulent strains of SF virus do not produce cytopathic effects in cultured mouse neuroblastoma cells (Atkins *et al.*, 1985), and the avirulent A7 SF virus mutant synthesizes less viral RNA in neuroblastoma cells than do virulent strains (Atkins, 1983). Mecham and Trent (1983) reported that, compared to TRD virus, TC-83 virus produced less virus-specific RNA and a higher ratio of 42S to 26S RNA during the first 4–8 hr postinfection in Vero cells. More infectious TRD virus than TC-83 virus was produced during the first 12 hr postinfection although maximum virus yields were similar (Mecham and Trent, 1983).

We previously reported a comparative sequence analysis of the 26S mRNA regions of the genomes of VEE TRD virus and its vaccine derivative, strain TC-83 (Johnson *et al.*, 1986). We now report the complete nucleotide sequences of the full-length genomic RNAs of both TRD and TC-83 viruses and summarize the nucleotide and deduced amino acid differences that in whole or in part are responsible for virulence and attenuation of VEE virus.

## MATERIALS AND METHODS

### cDNA synthesis and cloning

Plaque-purified VEE TRD and TC-83 viruses were used (France *et al.*, 1979). Genomic RNA was extracted from gradient purified virus and used as the template for synthesis of double-stranded complementary DNA (cDNA) using synthetic oligonucleotide primers as previously described (Kinney *et al.*, 1986). Viral cDNA was poly(dC)-tailed and cloned into poly(dG)-tailed pUC18 plasmid.

### Nucleotide sequencing

Cloned cDNA was excised from recombinant pUC18 plasmids with appropriate restriction endonucleases and subcloned into the filamentous bacteriophage M13mp18 or M13mp19. Partial exonucleolytic digestion of recombinant M13 DNA with bacteriophage T4 DNA polymerase (Dale *et al.*, 1985) resulted in nested cDNA deletions that permitted rapid sequencing of large cDNA clones without extensive restriction mapping (Chang and Trent, 1987). Sequencing was performed using the dideoxy chain termination method of Sanger *et al.* (1977, 1980). Dideoxy sequencing of heat-denatured, double-stranded recombinant plasmid DNA was performed as described by Chen and Seeburg (1985). Selected regions of VEE TRD and TC-

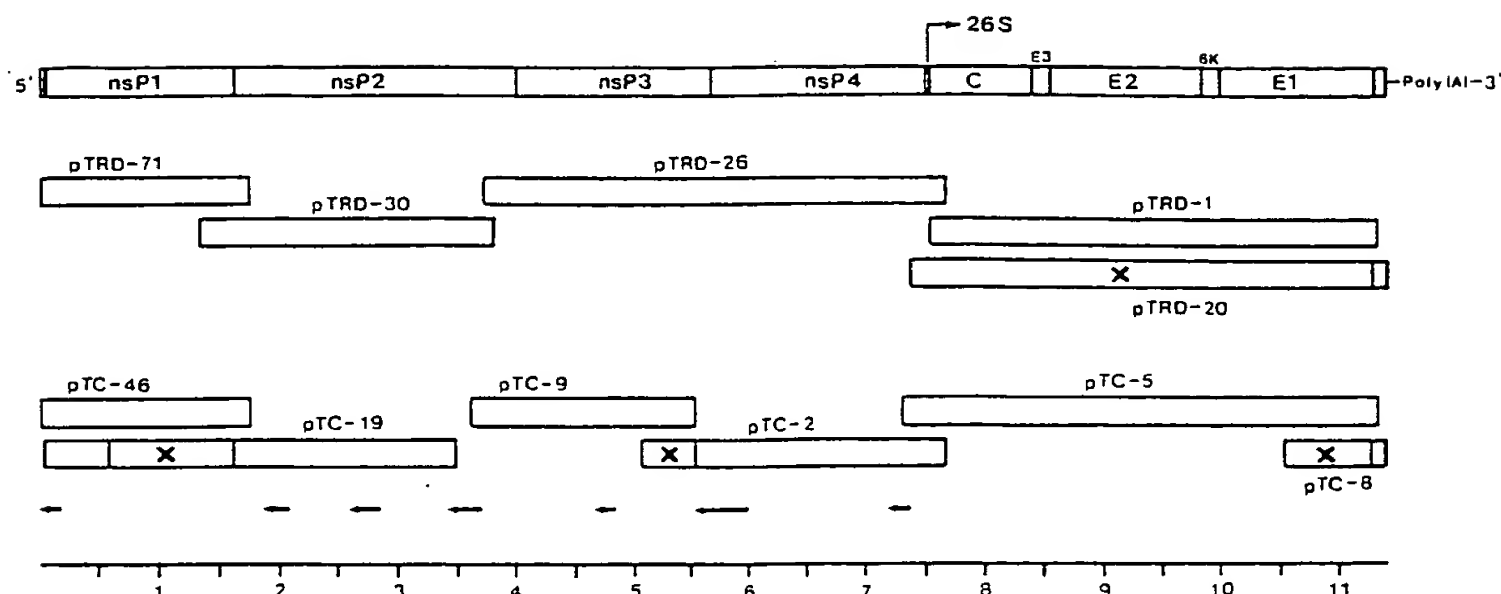


FIG. 1. Strategy for obtaining the complete nucleotide sequences of VEE TRD and TC-83 viruses. cDNA clones that were sequenced are indicated by open rectangles lacking the X symbol. Arrows indicate the extent of primer extension RNA sequencing. The organization of the genome is shown at the top, and genome position in kilobases is shown at the bottom.

83 genomic RNAs were sequenced by the dideoxynucleotide chain termination method (Johnson *et al.*, 1986). Incorporation of 7-deaza-2'-deoxyguanosine-5'-triphosphate (7-deaza-dGTP) in the dideoxynucleotide mixes enhanced sequence resolution in some areas (Barr *et al.*, 1986). Chemical sequencing, using 5'-end-labeled oligonucleotide primers (Maniatis *et al.*, 1982) was performed as described by Maxam and Gilbert (1977).

#### Computer analysis

For computer analysis of sequence information, we used a program (Kinney *et al.*, 1986) modified to run on IBM personal computers and the commercially available DNASIS<sup>2</sup> and PROSIS<sup>2</sup> software packages (Hitachi America, Ltd., San Bruno, CA).

## RESULTS

### Nucleotide sequences of VEE TRD and TC-83 viruses

The full-length nucleotide sequences of VEE TRD and TC-83 genomic RNA molecules were determined by sequencing both strands of five and six cDNA clones, respectively (Fig. 1). Regions that were not sequenced are indicated by rectangles containing the symbol X in Fig. 1. The nucleotide sequences of cDNA clones pTRD-1 and pTC-5 have been reported (Kinney

*et al.*, 1986; Johnson *et al.*, 1986). Clones pTRD-71 and pTC-46 containing 5'-end genomic sequences were obtained by priming first strand cDNA synthesis from genomic RNA templates with the oligonucleotide primer 5'-GTTTCAGCGAGAGGGTGGTGCAAG-3', the sequence of which was provided by data from clone pTRD-30. The sequence of a 150-bp region (between clones pTC-9 and pTC-19) that was absent from our genomic cDNA library of TC-83 virus was obtained by primer extension sequencing of TC-83 RNA.

The entire nucleotide and deduced amino acid sequences of the VEE TRD genomic RNA are shown in Fig. 2. Excluding the 5'-m<sup>7</sup>G cap, the TRD RNA genome was 11,444 nucleotides long with a base composition of 28.2% A, 24.5% C, 25.3% G, and 22.0% U.

### 5'-noncoding region of VEE 42S RNAs

In addition to cDNA clones pTRD-71 and pTC-46, three synthetic oligonucleotide primers (5'-TCAACGT-GAACTTTCTC-3', 5'-GGCTGTCTTCCTCGATG-3', and 5'-GTCATTATCAGTGACCTGCTTG-3') were used to obtain the 5'-terminal nucleotide sequences from RNA templates. These primers were complementary in sequence to the VEE 42S mRNA between nucleotide positions 48-152. Primer extension sequencing of the 5'-termini of TRD and TC-83 42S RNAs failed to resolve the 5'-terminal A nucleotide (Ou *et al.*, 1983) but did confirm the following 5'-terminal 19 nucleotides previously reported (Ou *et al.*, 1983). TC-83 differed from TRD virus in this region in the substitution of A for G at genome nucleotide position 3 (+ in Fig. 2). Excluding

<sup>2</sup> Use of trade names is for identification only and does not imply endorsement by the Public Health Service or by the U.S. Department of Health and Human Services.

the m<sup>7</sup>G cap, the 5'-noncoding region of the genomes of both viruses was 44 nucleotides in length.

### 3'-noncoding region

Clones pTRD-1 and pTC-5 lacked the terminal 65 or 66 nucleotides (excluding the poly(A) tail) of the 3'-noncoding regions of VEE TRD or TC-83 virus, respectively. We obtained the sequences of the 3'-termini by dideoxynucleotide primer extension sequencing of heat-denatured recombinant plasmids pTRD-20 and pTC-8 (Fig. 1). Our data confirmed the deletion of a U nucleotide in TC-83 virus at about genome position 11,405 as reported by Ou *et al.* (1982).

### 26S junction region

Sequencing of the 26S junction region of the 42S RNA where the genes encoding the nonstructural polypeptides end and those encoding the structural proteins begin on clones pTRD-26 and pTC-2, as well as RNA sequencing, showed that TRD and TC-83 nucleotide sequences in this region are identical. Thus, the single nucleotide difference between TRD and TC-83 viruses in the 26S junction region that we reported earlier is in error. The base at genome nucleotide position 7530 in both viruses is G.

### Translated region encoding VEE nonstructural proteins

Two in-frame AUG codons at nucleotide positions 12–14 and 45–47 occurred within the 5'-terminal 50 nucleotides of the VEE genomic RNA (Fig. 2). An out-of-frame AUG codon was present at the 5'-terminus in TRD genomic RNA, but not in TC-83 virus RNA due to the substitution of the A residue at nucleotide position 3 (Fig. 2). The AUG at nucleotide position 45–47 occurred within the sequence CAAA AUGG, which is the consensus sequence CAXX AUGG (where X = any nucleotide) for initiation codons in eukaryotic mRNAs (Kozak, 1981), and was chosen as the initiator of the nonstructural polyprotein precursor. Translation was terminated by an ochre (UAA) codon at positions 7524–7526. The deduced translated polyprotein was 2493 amino acids long. The open reading frame of the translated region was interrupted by an opal (UGA) termination codon at nucleotide positions 5682–5684. The nucleotide sequence of the region surrounding this opal codon was confirmed by dideoxy sequencing from RNA templates and by Maxam–Gilbert sequencing of single-stranded cDNA using radiolabeled primers 5'-GGGGC ATACGAAATCTCCAATT-3' and 5'-GAATACGTCTAGCTGTTATGGC-3', which were complementary to RNA nucleotide positions 5793–5815 and

5922–5943, respectively. The in-frame opal codon was determined to be present in the genomes of TRD and TC-83 viruses, as well as in two other VEE subtype 1AB virus strains, PTF-39 and 71-180.

### Deduced amino acid sequences of VEE nonstructural polypeptides

Nucleotide sequence data obtained from the cDNA clones (Fig. 1) revealed that the genes encoding the nonstructural proteins of VEE TRD and TC-83 viruses differed at genome nucleotide positions 1873 (T in TRD, A in TC-83), 4698 (A, G), 4809 (T, A), 5877 (G, A), and 7306 (T, C). To determine if these cDNA differences accurately represented nucleotide differences between TRD and TC-83 viral genomes, dideoxynucleotide sequencing of TRD and TC-83 42S RNA templates was performed with synthetic oligonucleotide primers. Nucleotide positions 1873, 4698, 5877, and 7306 were A, G, G, and U, respectively, in both viral RNAs. Thus, a T-A nucleotide difference at position 4809 encoding a Ser to Thr amino acid mutation was the only confirmed difference between the genes encoding the nonstructural polyproteins of VEE TRD and TC-83 viruses (+ in Fig. 2). This mutation occurred at nsP3 amino acid position 260. The nucleotide and deduced amino acid sequences of VEE TRD in Fig. 2 reflect the corrections obtained by primer extension sequencing of RNA.

### The nonstructural polypeptides of TRD, SIN, and SF viruses

The putative cleavage sites used to generate the nonstructural polypeptides from the polyprotein precursor were identified by comparing the VEE virus amino acid sequence with the amino acid sequences reported for SIN (Strauss *et al.*, 1984) and SF (Takkinen, 1986) viruses. The amino acid sequences of the translated nonstructural polyproteins of TRD, SIN, and SF viruses are compared in Fig. 3. Deletions (—) were inserted in order to optimize the alignment. Dots in the SIN and SF virus sequences indicate amino acid identity with TRD virus. The amino acids surrounding the polyprotein cleavage sites were highly conserved among the three viruses. The nsP1, nsP2, nsP3, and nsP4 polypeptides of VEE TRD virus showed 60–66%, 57–58%, 35–44%, and 73–71% sequence identity, respectively, with the cognate proteins of SIN and SF viruses. The nsP4 polypeptide was the most highly conserved of the four nonstructural proteins. When the nucleotide sequences of the translated regions of the three viruses were aligned, VEE sequences encoding nsP1, nsP2, nsP3, and nsP4 showed 60–63%, 57–



58%, 44–49%, and 65–63% identity, respectively, with cognate genes of SIN and SF viruses.

The deduced molecular weights of the nsP1, nsP2, and nsP4 polypeptides were approximately 60, 89, and 68–69 kDa, respectively, for VEE, SIN, and SF viruses. The 52 kDa weight of SF nsP3 was more than 8 kDa less than those of TRD and SIN viruses. Excluding VEE nsP3 amino acid positions 330–550, the nonstructural polyprotein structure was highly conserved when compared with SIN and SF viruses (Fig. 3). For example, 94% of 18 Trp, 73% of 73 Phe, 70% of 61 Cys, 70% of 120 Gly, and 69% of 116 Pro residues were conserved in the aligned nsP sequences of SIN and SF viruses (Fig. 3).

#### VEE nsP3 contains two peptide repeats

The nsP3 polypeptide, which was the least conserved of the four nonstructural polypeptides, had some interesting features. Although the amino terminal half of the molecule is highly conserved among VEE, SIN, and SF viruses, the carboxyl terminal half of VEE nsP3 showed virtually no sequence identity with the same region of SIN and SF nsP3 polypeptides (Fig. 3). The carboxyl terminal portion of VEE nsP3 contained a short repeated peptide segment (PXPAPRT, where X = variable position) and a long 34-residue peptide repeat: TPSXXPSRXXSRTSLVSPPGVNRVITREEXEAX (outlined and overlined amino acids in Figs. 2 and 3, respectively). These repeated peptide segments were identical in TRD and TC-83 cDNA clones and were confirmed by sequencing from RNA templates. SF nsP3 has a repeated peptide segment that is nearly identical to the first, short peptide repeat of VEE virus (Fig. 3) (Takkinen, 1986). Although the carboxyl portion of the nsP3 polypeptide of SF virus showed some identity to that of SIN virus, this region of SF nsP3 lacked 70 and 73 amino acids relative to VEE and SIN viruses, respectively. The in-frame opal termination codon (asterisk in Figs. 2 and 3) located six amino acid residues upstream from the carboxyl terminus of VEE nsP3 was aligned in Fig. 3 with the in-frame opal codon reported for SIN virus (Strauss *et al.*, 1983, 1984).

#### Summary of VEE TC-83 mutations

The nucleotide and deduced amino acid sequence differences between the genomes of VEE TRD and TC-

83 viruses are summarized in Table 1. TC-83 virus contained a single mutation in the 5'-noncoding region. The mutations in the E1 and E2 glycoproteins and the nucleotide deletion in the 3'-noncoding region of TC-83 virus have been reported previously (Johnson *et al.*, 1986; Ou *et al.*, 1982). The TC-83 nucleotide mutations are indicated in Fig. 2 by a + symbol. Effects of mutations on the hydrophobicity profiles of the E1 and E2 glycoproteins were discussed by Johnson *et al.* (1986).

## DISCUSSION

Extensive sequence conservation throughout the nonstructural polyproteins of VEE TRD, SIN (Strauss *et al.*, 1984), and SF (Takkinen, 1986) viruses highlights the hypervariability of the carboxyl terminal portion of nsP3 (Fig. 3). The lack of sequence conservation and the shortened sequence of the nsP3 polypeptide of SF virus raise questions regarding the functional significance of this region in virus replication. It is hypothesized that the in-frame opal termination codon near the carboxyl terminus of nsP3 in SIN, Middelburg, and RR viruses (Strauss *et al.*, 1983, 1984, 1988), and VEE virus perform a regulatory function by limiting translation of the full-length nonstructural polyprotein precursor to nsP4. SF (Takkinen, 1986) and O'Nyong-nyong (Strauss *et al.*, 1988) viruses, on the other hand, have no in-frame terminator in nsP3. The alphavirus nsP4 polypeptide is the most conserved of the nonstructural proteins, and therefore must play a vital role in virus replication. Alphavirus polypeptide nsP1, nsP2, and nsP4 share three domains of sequence homology with the replicases of three plant viruses, and the T2 polypeptide of tobacco mosaic virus is also translated by read through of a termination codon (Strauss and Strauss, 1986). The infectious Sindbis virus cDNA clone (Rice *et al.*, 1987) may be used to analyze the functional roles of defined regions in individual alphavirus proteins, such as the Gly-Asp-Asp amino acid triplet located at nsP4 amino acid positions 464–466, that may be a functional domain in the RNA polymerase of positive strand RNA viruses (Kamer and Argos, 1984; Takkinen, 1986).

The nucleotide sequence information in Fig. 2 contains two corrections to our earlier reports (Kinney *et*

FIG. 2. Nucleotide sequence of the VEE TRD 42S genomic RNA and the deduced amino acid sequence of the encoded polypeptides. The single letter abbreviation is aligned with the first nucleotide of codon. Arrows indicate proteolytic cleavage sites of the translated polyproteins and the putative 26S mRNA start site. The overlined amino acid sequences represent probable transmembrane domains of the E1 and E2 envelope glycoproteins. Amino acid repeats in nsP3 are outlined. The in-frame opal termination codon near the carboxyl terminus of nsP3 (\*) and the confirmed nucleotide mutations (+) in TC-83 virus are indicated. Potential glycosylation sites in E1, E2, and E3 are indicated by solid circles.



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*al.*, 1986; Johnson *et al.*, 1986). First, the 26S junction regions of TC-83 and TRD viruses are identical. Second, the sequence information in a compressed area at the 3'-terminus of the 6K gene was resolved more clearly by Maxam-Gilbert sequencing of single-stranded cDNA synthesized on RNA templates. The carboxyl terminus of the 6K polypeptide is Ala-Gly-Ala, not Ala-Pro-Ala as was reported earlier.

Attenuation of a virulent virus may result from mutations in the virus structural proteins which affect viral morphogenesis, host cell tropisms, and interaction with the host immune system, or from mutations in nonstructural proteins or noncoding regions which affect virus replication. Comparison of the entire genomes of TRD and TC-83 viruses reveals only 11 nucleotide changes (0.1%) in the 11,443-nucleotide genome of the attenuated TC-83 derivative. The total number and distribution of TC-83 mutations contrasts with the 17D-204 yellow fever vaccine in which 68 nucleotide mutations relative to the virulent Asibi strain of yellow fever virus were distributed throughout the genome (Rice *et al.*, 1985; Hahn *et al.*, 1987). The single nucleotide mutations in the 5'- and 3'-noncoding regions of TC-83 virus confirm the results reported by Ou *et al.* (1982a, 1983). Reversion to virulence of polio type 3 vaccine virus has occurred via a single point mutation in the 5'-noncoding region of the genome (Evans *et al.*, 1985). Recent sequence analysis of the genomes of mouse-avirulent (RRV NB5092) and mouse-virulent (RRV T48) strains of RR virus detected 284 nucleotide differences between the two genomes, as well as deletions or insertions in the noncoding regions. Because 12 and 36 substitutions occurred in the translated structural and nonstructural proteins (Faragher *et al.*, 1988), respectively, and a large number of nucleotide differences occurred throughout the genomes of these two viruses, study of the molecular basis of virulence is complicated for this virus pair.

Although the mutations in the noncoding regions of TC-83 virus and the single conservative Ser to Thr substitution in the nonstructural polyprotein may contribute to attenuation, the dearth of mutations in the 5'-two-thirds of the genome enhances the significance of the amino acid substitutions in the E1 and E2 envelope glycoproteins of this virus (Johnson *et al.*, 1986). The nonconservative nature of the five amino acid substitutions in the E2 glycoprotein makes them promising as

molecular determinants of virulence. However, reversion of a mutation affecting the isoelectric point of the E1 glycoprotein of a temperature-sensitive, avirulent mutant of VEE virus occurred concomitantly with reversion to virulence (Emini and Wiebe, 1981). Lustig *et al.* (1988) determined that specific loci in both E1 and E2 proteins of SIN virus were important for neurovirulence in adult mice and that a gradient of virulence, depending on the E1 and E2 loci involved, existed for neonatal mice. Analyses of recombinant polio type 1 viruses obtained from infectious cDNA clones demonstrated that loci involved in attenuation were distributed in several regions of the genome and that alterations in antigenicity correlated poorly with virulence changes (Omata *et al.*, 1986).

Although loss of epitopes involved in neutralization of virulent virus has been associated with attenuation (Dietzschliid *et al.*, 1983), this does not appear to be the operative mechanism in attenuation of the TRD virus. Of the 12 epitopes identified on the envelope glycoproteins of VEE virus, only E2<sup>a</sup> is TC-83-specific. This epitope is defined by a monoclonal antibody that does not inhibit hemagglutination or neutralize virus infectivity. Eleven epitopes, including those involved in virus neutralization, are conserved in both TRD and TC-83 viruses (Roehrig *et al.*, 1982; Roehrig and Mathews, 1985). Monoclonal antibodies that define VEE epitopes close to the E2<sup>c</sup> neutralization epitope are most efficient at blocking virus attachment *in vitro* (Roehrig *et al.*, 1988).

Sequence analyses of mutant SIN viruses that were selected for rapid penetration *in vitro* and were attenuated for neonatal mice demonstrated a Ser to Arg substitution at E2 amino acid position 114 (Davis *et al.*, 1986). The attenuated SIN mutant elicited monoclonal antibody that preferentially neutralized the mutant virus as opposed to the virulent parent, although the antibody reacted similarly with both attenuated and parent viruses in binding assays (Olmsted *et al.*, 1986). Recently, Johnston and Smith (1988) obtained seven rapid penetration isolates of VEE TRD virus that were avirulent for adult mice. Two of these were also avirulent for hamsters. Thus, at least two glycoprotein domains that are involved in virus penetration *in vitro* define virulent and attenuated phenotypes and altered tissue tropism or virus-tissue interaction.

FIG. 3. Comparison of the deduced amino acid sequences of the nonstructural polyproteins of VEE TRD virus with those of SIN (Strauss *et al.*, 1984) and SF (Takkinen, 1986) viruses. Deletions (—) were introduced to optimize the alignment. Solid dots in SIN and SF sequences indicate identity with VEE virus. Cleavage sites are indicated as are the amino acid repeats (solid overlines). The in-frame opal termination codon near the carboxyl terminus of VEE nsP3 is shown (\*).



VEE	HE--KVHVDI EEDSPFLRALORTFPQFEVAKQVTONOHANARAF SHLASAL IETEVUPSDI--LDIGSAPARRMYSHKHAYHCICPMHCAEDUPURLYTA	NS1	97
SIN	..KPV..N..VDPQ...VVO...KS...V.Q...P...L...PTTA...F.E.Q...V...SP...MM...	NS1	99
SF	..AA...A...IKS...KA...S...SL...P...T...Q.T.K...L...S...M.T...V...S...E...DS...	NS1	98
VEE	TKLK-KNCKE I TOKELDKMKELAAVMSDPOLETETMCLHODESCRYEQGVAVYQVAVOCPTSLYHQANKGVRYAYWIGFOTTPFMFKNLGAYPSYS	NS1	196
SIN	S...AE.A...N.N.HE..I.D.RT.LDT...A..PSL.F.N.VT.NMRAEYS.M...-INA.GT1...M...TL...Q...SAM...S...A.N	NS1	197
SF	K...A-AASGKVL.R.IAG.I.TD.QT..AT..A.SP.F...T.VT..TAAE...HA...M...T...DA...T.A	NS1	197
VEE	TNWADETULTARNICLSSDVMERSRRGMSI LRKKYKLPNNVLF SVGSTIYHEKROLLRSWHLPSVFLHRGKNYTCRCETIVSCDGYVVKRI AISPLG	NS1	296
SIN	...K...E...TKLS.CRTGKL..M...E...GSR.Y...L.P.H.AS.Q...N...S...D.V...E...K.T...I	NS1	297
SF	...Q...Q...AASLT.GRLGKL...Q...COT.M...L.T.S.K...K...SF...D...E...K.TMC...	NS1	297
VEE	YGKPSGYAATMHREGFLCCKVTDLTNGERSVPVCTYVPATLCQDMGTI LATDVSADDAQKLLVGLNQRIVVNGRTQRNTNTMKNYLLPVVAQAFARWAK	NS1	396
SIN	T.ETV...V.HNS...L...VK...I...I...M...I.P...I...N...Q...I...G.SK...	NS1	397
SF	...TV...V.Y.A...V...T...VK...S.I...TPE...I...V...SK...R	NS1	397
VEE	EYKEDQEDERPLQLRORQLVMGCCWAFRRHKITSYKRPOTQTI I KVNDSFHSFVLPRIGSNTLE IGLRTRIRKMLEEHKEPSPLITA-EDVOEAKCAAD	NS1	495
SIN	..R.D.LDN.KM..T.E.K.TY..L...TK.VH.F.RP.G...CV..PAS.SA.PHSSVTT.S.PMS..QKLKLA.QPK..EKL.QVSE.L.M...A.FE	NS1	497
SF	...A.LD..K...V.E.S.TCC.L...KTR.MHTM..K...P.E.N...I.SLW.TG.A.PV.S...LAK.TKRE..PVLDASSA-RO.EQ	NS1	495
VEE	ERKEVREAEELRAALPPLAAD-VEEPTLEADVOLM-LQ-CAGAI GSVETPRGLIKVTSYOGEDKIGSYAVLSPOAVLKSEKLSCHPLAEQVIVI THSGR	NS2	56
SIN	DAQ.EAR..K..E...V..KGI.AAA.VVCEVEG..ADI...AL...HVR.II.PQANDRM..Q.I.V..NS...NA..APA...D..KI...	NS2	56
SF	..E..RL...LT.E...VPIAPA..GVV...VEE.EYH...V...SAL...AQPNVLL.N.V...T...S..APV...KI...N...	NS2	56
VEE	KGRYAVEPYHKGKVVPECHAI PVQDFQALSESATIVYNEREFVNRYLHIIATHGCAINTDEEYKTVKPESEHOGEYLYDIORQKCVKKELVTLGLTGL	NS2	156
SIN	S...DA..LM.A.G.V.WPE..L...K.Y...M..P.K..E..Q..VT.AELAEI..VF.V.K.R...EAS..V.S...	NS2	156
SF	A.G.Q.DG.D.R.LL.C.S...PE...M...K.Y...V..PS...N.EK.RAERT.A..VF.V.K.C...R.EAS..V.V...	NS2	156
VEE	VDPPEHEFAYESLRTPAAPYQVPTIGVYCVPGSGKSGI I KSAVTKKDI.VVSAKKENCAEI I ROVKKMKGLDVNARTVDSVLLNGCKHPVETLYIDEAFA	NS2	256
SIN	TN..Y..L.L.G.K...V..K.E...I..T...A...T..AR...T.G...R..EA..LRLR.MQITSK...M...HKA..V..V...	NS2	256
SF	TN...G.KI..S...KTTVV..F...A...L...H...T.G...Q..VN...HR.KCTSRENS..I...RRA.DI..V...	NS2	256
VEE	CHAGTLRALIAIIRP-KKAVLCGDPKQCGFFNMMLKLVHFNH---EICTQVFHKSISRCTKSVTSVSTLFYOKKMRITNPKETKIVIDTTGSKPKQD	NS2	352
SIN	...A.L...V..R..V...M...Q...PEKD...KT.Y.Y...QP..AI...H..G..K...CKKN.E..I..A...PG	NS2	356
SF	..S...L...LVK.RS.V...Q...N...---N...E.C...RP..AI...H.GG...CNKP..I...Q...PG	NS2	353
VEE	DLILTCFRGWKQLQIDYKGNEMTAASQGLTRKGVYAVRYKVNENPLYAPTSEHVNVLLTRTEDRIVWKTLAGDPWIKTLTAKYPCNFTATIEEWQAE	NS2	452
SIN	..I...A...L...P.H.V...Q...I...L...Q...QP.NIPK...Q...D.E...	NS2	456
SF	..IV...A...L...R.H.V...Q...A...V.SNIPQ...L...E...	NS2	453
VEE	HDAIMRHILERPDP-TOVFONKANVCHAKALVPVLKTAGIOMTTEQWNTVDY-FETDKAHSAEIVLNQLCVRFGLDLOSLFSAPTVPISI-----R-	NS2	543
SIN	..KG.IAA.NS--T.RANP.SC.T...E..I..A...VL.GC..SELFPQ.AD..P..IYA.DVI..K...M..T...KQSI..TYHPAOSA.P	NS2	555
SF	..K..KV.EG--AAPV.A...S...D...RL.A.E.S.IITA.KE.R.Y.PVVA..EI.TKYV.V...K.S.YY---E---	NS2	545
VEE	NNHWONSPSPNMYGLNKEVVRQLSRYPQLPRAVATGRVYDMNTGTLRNYOPRI NLVPVNRRLPHALVLLHNEHPQSDFFSVSKLGRTVLVVGE-KLS	NS2	642
SIN	VA...GTRK..YDHAIAAE...F..VFQLAGK.TQL.LQ..RT.VISAQH...N...PEYK.KQPGPVKK.LNQF.HHS...S.E.IE	NS2	654
SF	.....R.GGR...F.AATAAR.EA.HTF.KQGW..KQAVIAERKIQPLSLVD.VI.I...AEYKTVKG.RVEWL.N.VR.YH..L.S.YN.A	NS2	645
VEE	VPGKMVDWLSRPEATFRARLDL--GIPGDVPKYDI I FVNVRTPYKYHHYQOCEDHAI KLSMLTKKACLHLNPGCTCVSICYGYADRASEI I CAIARQF	NS2	740
SIN	A.R.RIE.IAPIGI.GADKNYN.AF.F.PQA-R..LV..I..IG.K.RN..F...AT.KT.SRS.LNC...L.VKS...N..DVVT.L..K..	NS2	753
SF	L.RRR.T...PLNVTGADRCY..SL..L.A.AGRF.LV...IH.EFRI...V...M..Q..GCD.LRL.K...ILM-RA...KI...AVVSSLS.K.	NS2	744
VEE	KFSRVCKPKSSLEETEVLVFIYGYDR-KARTHNPYKLSSTLTNIYTGSRLEACG APSYHVVRGDIATATEGVI I NAANSKGQPGCGVCGALYKFPES	NS3	44
SIN	VRVSAAR.DCVSSN..MYLI.RQL.NSRT.QFT.HM.NCVISSV.E.T.-OCV.A...RTK.EN..OCQ.EAVV...PL.R..E...R..I..RW.T.	NS3	44
SF	SSA..LR.DCVTSN...FLL.SNF.NG..-PSTLHQMNTK.SAV.A.EAM.T...I...R.K.A...C..AAVY...AR.TV.D...R.VA..W.SA	NS3	44
VEE	FDLQPIEYCKARLVKGAAKHIIHAVGPNFNKVEVEGDKQLAEAYESI AKIVNONNYKSVAIPLLSTGIFSGNKORLTQSLNHLTLALOTTOADVAIYCR	NS3	144
SIN	..TDSAT.T.T..MTVCLG.KV...D.R.HP.A.AL.L.QN..HAV.DL..EH..I...YAAG...EV...C.T...R...T...L	NS3	144
SF	..KGAATP..TIKT.MCGSYPV...A..SATT.A..RE..AV.RAV.AE..RLSLS...V...GR...Q...F..M.A...T...	NS3	144
VEE	DKKWEMTLKEAVARREAVEEICI SDSSVTEPDAELVVRHVPKSSLAGRKGYSTSGKTFSYLEGTFKHQAADIAE INAMWPVATEANEQVCMYILGSM	NS3	244
SIN	...KERIDA.LQLK.S.T.LK--E--DM..I..D...WI..D.C.K...F..TK..LY..F...M...KVLF.NDQ.S...L.A...T...	NS3	240
SF	...S..KKIQ..IDM.T...LLN---ELTT-D...D...V...T..SLY..F...N...I.M...LTL..RLQ...RI.L.A...T...	NS3	239
VEE	SSIRSKCPVEESEASSPPSTLPCLCIHAMTPERVORLKASRPEQITVCSSFLPKYRITGVQKIQCSQPI I FSPKVPAYIHPRKVLVETPPVDETPEPSA	NS3	344
SIN	EA..E...DHNP.S...K...MY...H...RSNNYKEV...T...HK.KN...V..TKVV..N.HT..FVPA...IEVPEOPTAP.AQAE	NS3	340
SF	DN.G...ND.QS.T..R.V...RY..A..IA..RSHQVKSVM...HVD...VK.EKVL..D.T..SVVS...AAS.TDHSRSLRGF	NS3	339
VEE	ENQSTEGTPEOPPLI TEDETTRITPEPI I EEEEDSI I SLLSGPHTHOVLQVEADHGPSSVSSSSWSI PHASOFVOSLSI I DTLGASVTSGATSAET	NS3	444
SIN	..APEVVA..SPSTADNTSLDV.DISLDMOOS.GSLFS.FSGSDNSI I TSMOSWSSGSSLE I VORROVVVADVHAVQEPAP.PPPRLKKMARLA.ARK.P	NS3	440
SF	DLDW.TOSSSTASOTMSLPSLQSCDIOS.Y.PMAPIVVTADVHPEPAGIADLA..V..PE.ADHVLEN-----P.PPPRPKRAAYLASRA..	NS3	426
VEE	NSYFAKSMELARPVPAPRI VFRNPPHPAPRI I TPLAPSACRSRTSLVSTPPGVNRVI I TREELEALTSPRTPSRSVSRTSLVSNPPGVNRVI I TREEFEA	NS3	544
SIN	TPPASN.S.S.HLSFGGVMSLCSI I FOGETARQAAVQPLATGPTDVPMSFGSFGCEI I DELSRVTESEPVLFCSFEPCEVNSI I SSRSAVSFPLRKORR	NS3	540
SF	-----K--T...AFRNLK-----P.TFGDFDEME.DALASCI-----FGDFDDVLR-----	NS3	476
VEE	FVAQQQ---*RFDACA I YIFSSDTGQGHLOOKSVROTVLSEVVLTERTELE I SYAPRLDOCKEELLRKKLQNLNPTANRSRYOSRKVENMKAI I TARRILOG	NS4	83
SIN	RRRSRTTEY.LTGV.G...T...P...K...L.NQ.T.PT...NV..RIH..V..FS...Q.KLRY.MM..E..K...E.L.S...	NS4	83
SF	...LGR...HN.QCAQ.DAVQE.KM.P.K..T.R.K..LL.M.MM..SE..K...TVVO.LTS.	NS4	83
VEE	LGHYLKAECKVEE-YRTLHPVPLYSSSVNRAFSSPKVAEACNAMLKENFPTVASYCI I PEYDAYLDHVDGASCCLDTASFCAKLRSPKXHSYLEPTI	NS4	182
SIN	..RL.NS.TDQP...KITY.K...PANY.D.QF..AV..NY.H..Y...Q.TD...IVA...T...E.RA.N...	NS4	182
SF	ARL.TG.DVGRIPT.AVRY.R.V..PT.IER...D..IA...EY.SR.Y...Q.TD...SDT...R.T...CY..H.A.HQ..V	NS4	183
VEE	RSAYPSAIQNTLQNVLAATKRNCNVTOHREL PVLDSAAFNECFKKYACNNEYWETFKENPIRLTEENVNYI I TKLKGPKAAALFAKTHNLNMLDIPM	NS4	282
SIN	...M...T...D...R...D...E.ARK...I.T.F.TA.VAR...Y..VP..EV...	NS4	282
SF	...PF...TM...V...R...SG...EYAKQ...I.T..ITT.V...VP..EV...	NS4	283
VEE	DRFVMOLKROVKVTPGKHTERPKVQVIAADPLATAYLCGIHRELVRRLNAVLLPN I HTLFOMSAEDFOAI I AEHFQPGDCVLETDIASFDKSEDDAM	NS4	382
SIN	...M...E...T...KQ..P...Q...Q...SL	NS4	382
SF	...TV.M...E...R..V...S..H...P...Q...SL	NS4	383
VEE	ALTALMILEDLGVAELLTLIEAAFGEISSIHLPTKTKFKFGAMMKSCMFLTLFVNTVINIVIASRVLRLRTGSPCAAF I GDDNIVKGVKSOKLMAORC	NS4	482
SIN	..G...QP..D..C...T...G.R...L.V...E..KT.R...H..V...E..E...	NS4	482
SF	..G...QY..D...C...G.R...I..L.T...EQ..D.A...H..I...E...	NS4	483
VEE	ATWLNMEVKI I DAVVGEKAPYFCGGF I LDCSVTGTACRVADPLKRLFKLGKPLAAODEHODRRRALHEESTRWNRVGI I SELCKAVESRYETVGTSI I V	NS4	582
SIN	..S.V...I..RP...Q...S...P...E..Q...TGT.AV..TT...VDNITPVL	NS4	582
SF	..S.V...M..P...VF...Q...S...T.E.KQ.E...SD.VSK.F.T.LGA..EV.LT...VE.CKS.L	NS4	583
VEE	MAMTTLASSVKSFSYLRCAPITLYG	NS4	607
SIN	L.LR.F.Q.KRA.QAI..EIKH...GPK	NS4	610
SF	I...RDI..A.KK..PV.H...GPRLV	NS4	614

TABLE 1

NUCLEOTIDE AND DEDUCED AMINO ACID DIFFERENCES BETWEEN  
VEE TRD AND TC-83 VIRUSES

Position		Nucleotide		Amino acid change	
Nucleotide	Amino acid	TRD	TC-83	TRD	TC-83
3	5'-noncoding	G	A	Noncoding	
4,809	nsP3-260	U	A	Ser	Thr
8,584	E2-7	G	U	Lys	Asn
8,816	E2-85	C	U	His	Tyr
8,922	E2-120	C	G	Thr	Arg
9,138	E2-192	U	A	Val	Asp
9,397	E2-278	U	C	None	
9,450	E2-296	C	U	Thr	Ile
10,478	E1-161	U	A	Leu	Ile
10,630	E1-211	A	U	None	
11,405	3'-noncoding	UU	U	Noncoding	

Although we have defined a set of mutations that are associated with the attenuated phenotype of TC-83 virus, the relative contribution of each of the mutations remains to be assessed. The most direct way to define the functional role of these changes lies in recombination experiments utilizing infectious cDNA clones of VEE virus (work in progress). Sequence analysis of rapid penetration mutants and variants selected under the pressure of monoclonal antibodies should also prove invaluable in defining protein domains that may frequently be involved in alterations of virulent and attenuated phenotypes.

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